Molecular Epidemiologic Trends of Diarrhea-Causing Virus Infection From Clinical Specimens in Cheonan, Korea, in 2010–2012

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Background: The enteric viruses have high health significance in humans, ranging from poliomyelitis, hepatitis, and gastroenteritis to innocuous infections, and the human enteric viral infection is the one of the most common diseases during early childhood. This study was conducted from July 2010 to October 2012. **Methods:** We subjected 788 stool specimens using multiplex polymerase chain reaction (mPCR) tests that could simultaneously detect five enteric viruses, group A rotavirus (GAR), enteric adenovirus (EAdV), norovirus GI (NoV-GI), norovirus GII (NoV-GII), and human astrovirus (HAstV). The data were analyzed according to seasonality and patient age and sex. **Results:** Two hundred and seventy specimens (34.3%) were positive,

276 viruses were detected in the 788 sample. The prevalence of GAR, EAdV, NoV-GI, NoV-GII, and HAstV infections in the 270 mPCR-positive specimens was 101 (36.6%), 28 (10.1%), 4 (1.4%), 132 (47.8%), and 11 (4.0%), respectively; six specimens (2.2%) contained double infections. NoV-GII and GAR infections occurred mainly in the winter and spring. **Conclusion:** We described the epidemiological analytic data of the diarrhea-causing viruses in the population of local society of Korea. These results could be helpful for the diagnosis and subsequent epidemiological surveillance of enteric viral infections. J. Clin. Lab. Anal. 28:47–51, 2014. © 2013 Wiley Periodicals, Inc.

Key words: multiplex PCR; season; ages; multiple infection; diarrhea

INTRODUCTION

Despite improvements in sanitation levels and lifestyles, viral gastroenteritis with diarrhea affects many people around the world, especially children, infants, and toddlers (1). Furthermore, although the number of deaths due to viral gastroenteritis with diarrhea has been steadily decreasing in developed countries due to improved sanitary environments, the incidence and mortality rates in developing countries remain high (2, 3).

The causative pathogens of viral gastroenteritis with diarrhea, which are of increasing importance, include group A rotavirus (GAR), enteric adenovirus (EAdV), human astrovirus (HAstV), and norovirus (NoV) along with other viral pathogens including torovirus, picobirnavirus, picornavirus, and enterovirus 22 (1, 4–6).

Traditional tests to identify pathogens causing diarrhea, including electron microscopy, enzyme-linked immunospecific assay, and stool culture, require experienced examiners and often involve issues relating to sensitivity,

and false positive results may occur. Moreover, stool culture results require 2–3 weeks. Recently, a molecular diagnostic technique based on multiplex polymerase chain reaction (mPCR) has been used clinically for rapid diagnosis (7, 8).

The purpose of this study was to assess the relative importance of each type and species of viral pathogen in viral gastroenteritis with diarrhea. We performed mPCR, which allows the simultaneous amplification of more than one target nucleic acid in a single test tube, on five viral pathogens: GAR, EAdV, NoV-GI, NoV-GII, and HAstV.

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We tested patient stool samples to detect viral infections, carried out a survey of prevalence trends, and conducted surveillance by analyzing the distribution of incidence based on sex, age, and temporal distribution in order to generate foundational data for establishing laboratory diagnostic systems and preventative measures.

MATERIALS AND METHODS

Clinical Samples for mPCR Analysis

Between July 2010 and October 2012, 788 clinical stool specimens were consecutively collected from patients and submitted to Dankook University Hospital for routine diarrhea-causing viral screening. The collected stool specimens were stored at 4°C and nucleic acid extraction was attempted within 24 h.

Multiplex RT-PCR Analysis

PCR was performed using the Seeplex diarrhea virus detection kit (Seegene Co., Seoul, Korea), according to the manufacturer's instructions, with a PTC-200 PCR system (MJ Research, Hercules, CA). An mPCR assay was designed to detect the diarrhea-causing viral pathogens as follows: open reading frame 1a (ORF1a) of HAdV that encodes the nonstructural polyprotein 1a as a viral protease and an RNA-dependent RNA polymerase, the *vp4* gene of GAR that encodes the protease-sensitive glycoprotein VP4 of GAR genotype P, the *hexon* gene of EAdV that encodes the hexon coat protein found in adenoviruses, and ORF2 of NoV-GI/GII that encodes the major capsid protein of NoV called VP1. The internal mPCR control was the *Cesa3* gene of *Arabidopsis*. The mPCR for the five viruses was performed using a Seeplex[®] diarrhea ACE detection kit with dual specificity oligonucleotides (Seegene Co.) as primers, and the five pathogens were detected in a single test tube (Table 1).

TABLE 1. The Target Genes for Diarrhea-Causing Viruses

Target viruses	Target region	Target protein	Gene size (bp)
<i>Arabidopsis</i> (Internal control)	<i>Cesa3</i>	Glycosyltransferase	1,000
Human astrovirus	ORF1a	Nonstructural proteins	650
Group A rotavirus	<i>VP4</i>	Outer capsid protein	541
Enteric adenovirus	<i>Hexon</i>	Viral capsid protein	411
Norovirus GI	ORF2	Viral capsid protein	304
Norovirus GII	ORF2	Viral capsid protein	214

Distilled water (1 mL) was added to the stool specimen, and RNA was extracted from a 200 µL sample using a QIAamp MinElute virus spin kit (Qiagen, Hilden, Germany). cDNA was synthesized from the extracted RNA using a RevertAid First Strand cDNA synthesis kit (Fermentas, Ontario, Canada). cDNA was synthesized in a 20 µL reaction containing 1 µL of random hexamer, 8 µL of total RNA, 1 µL of reverse transcriptase, 1 µL of RNase inhibitor, 2 µL of dNTP, 4 µL of 5× reverse transcription buffer, and 3 µL of distilled water at 37°C for 90 min. The obtained cDNA was used for the mPCR.

The mPCR was performed using a PTC-200 PCR system. The reaction conditions were 30 s at 94°C, 90 s at 60°C, and 90 s at 72°C for 40 cycles. At the end of the cycle, the reaction was left to stabilize at 72°C for 10 min.

The mPCR products were electrophoresed in a 2% agarose gel with ethidium bromide for 30 min at 100–150 V. The agarose gel was rinsed with distilled water, amplification was assessed by visualizing the gel on a UV transilluminator, and the results were analyzed after a photograph was taken (Fig. 1). Using the mPCR detection results for the diarrhea-causing viral pathogens, we performed statistical analysis of the various factors within

Fig. 1. The PCR products were resolved by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining. (A) 100 bp molecular size markers, (B) GAR- (541 bp) and NoV-GII-positive (214 bp), (C) GAR-positive (541 bp), (D) NoV-GII-positive (214 bp).

Fig. 2. There were 267 viruses from patients between the ages of 0 and 9 years. Of these, 98 viruses (36.7%) were isolated from individuals aged 1–2 year, 84 viruses (31.5%) from those aged 0–1 years, and 31 viruses (11.6%) from those aged 2–3 years.

the viral infection patterns such as multiple infection, sex, and temporal distribution.

RESULTS

In the 788 specimens, 270 (34.3%) were PCR-positive for the targeted viral pathogens, and 276 different isolates were detected. In the PCR-positive patients, 164 were men and 106 were women, and the male–female ratio was 1.55:1. Age distribution analysis showed that of the 270 PCR-positive individuals, 261 (96.7%) were aged 0–9 years. We detected 276 isolates: 267 (96.7%) were from patients between the ages of 0 and 9 years, and of these isolates, 47.9% (128/267) were NoV-GII and 36.3% (97/267) were GAR (Fig. 2). Among the patients aged 0–9 years, those aged 1–2 years had the highest PCR positivity at 54.9% (96/175); this age group comprised 35.6% (96/270) of the 270 PCR-positive patients.

In the 276 isolates, NoV-GII was most frequently detected (47.8%; 132/276), followed by GAR at 36.6% (101/276), EAdV at 10.1% (28/276), HAstV at 4.0% (11/276), and NoV-GI at 1.4% (4/276). Six (2.2%; 6/276) of the PCR-positive specimens were positive for two viral types. No specimens were positive for three or more viral types. Twelve isolates (4.3%; 12/276) were detected in the six specimens positive for two pathogens, and GAR was most frequently detected (1.8%; 5/276; Table 2).

TABLE 2. Number of Detected Viruses According to Infection Types

Positive	Single infection (%)	Double infection (%)	Total (%)
Group A rotavirus	96 (34.8)	5 (1.8)	101 (36.6)
Norovirus GI	3 (1.1)	1 (0.4)	4 (1.5)
Enteric adenovirus	26 (9.4)	2 (0.7)	28 (10.1)
Human astrovirus	11 (4.0)	0 (0.0)	11 (4.0)
Norovirus GII	128 (46.4)	4 (1.4)	132 (47.8)
Total	264 (95.7)	12 (4.3)	276 (100.0)

The monthly PCR-positive rate was highest in December (62.5%; 40/64), followed by February (58.3%; 42/72) and January (54.1%; 33/61). For NoV-GII, 132 isolates were detected; there were 32 detections in December and 21 in January. GAR was detected most frequently in April, when it was detected in 28 samples, followed by 22 samples in February. NoV-GII and GAR were more frequently present in samples collected during the winter and early spring (Table 3).

DISCUSSION

This study was conducted to assess the infection status of pathogens causing viral gastroenteritis and the distribution patterns of diarrhea-causing endemic viruses. The

TABLE 3. Monthly Distribution of Positive Specimens for Diarrhea-Causing Viruses

Month	Number of viruses detected	GAR	NoV-GI	EAdV	HAstV	NoV-GII	Number of patients	Number of positive patients	Positive rate (%)
3	37	21	0	1	0	15	71	35	49.3
4	40	28	1	1	1	9	86	40	46.5
5	11	4	0	2	3	2	71	11	15.5
6	9	4	0	3	1	1	57	9	15.8
7	12	6	0	1	1	4	93	12	12.9
8	7	0	0	1	1	5	63	7	11.1
9	10	2	0	4	2	2	67	10	14.9
10	8	0	0	2	0	6	40	8	20.0
11	23	2	1	5	0	15	43	23	53.5
12	41	4	1	3	1	32	64	40	62.5
1	35	8	1	4	1	21	61	33	54.1
2	43	22	0	1	0	20	72	42	58.3
Total	276	101	4	28	11	132	788	270	34.3

study was conducted in 2 years and 4 months between July 2010 and December 2012 at Dankook University Hospital in Cheonan, Korea, and involved patients suspected to have virus infection. In the 788 specimens tested, 270 were positive, yielding 34.4% PCR positivity, and 276 virus isolates in total were detected. According to the Korea Centers for Disease Control and Prevention (KCDC, (9)), the PCR positivity of diarrhea-causing viruses in 2006 and 2007 was 19.6% and 24.2%, respectively, and the average PCR positivity between 2006 and 2010 was 21%. In comparison, the PCR-positive rate was higher in this study. We believe that this was due to a difference in the testing methods, and further investigation is necessary.

The male–female ratio for virus-positive patients was 1.55:1, similar to the ratio of 1.22:1 reported by Chung et al. (10) and 1.5:1 reported by Nguyen et al. (11).

The average age of the 270 PCR-positive patients was 3.3 years, and 96.7% (261/270) of them were aged between 0 and 9 years. Among the individuals aged 0–9 years, 96 were aged between 1 and 2 years, making up 35.6% (96/270) of the total number of patients; the PCR positivity of those aged 1–2 years was the highest at 54.9% (96/175). Chung et al. (10) determined that positivity in those aged 0–10 years was 92.9%, and that positivity in the group aged 1 year and under was the highest at 56.8%. These results are also consistent with those reported by Brandt et al. (12) and Giordano et al. (13). We believe that these results are due to younger patients having lower immunity to viruses or being less careful about viral infections.

In the 276 virus isolates detected, the most frequently detected were NoV-GII (47.8%; 132/276) and GAR (36.6%; 101/276). In a similar report by the KCDC, GAR (11.2%) was most frequently detected in 2006. However, in the same report, NoV-GII detection was high at 10% or more between 2007 and 2010, with the exception of 2009.

Furthermore, the NoV-GII isolation rate differed greatly from that of our study, demonstrating that the detection rate of NoV has been increasing, thus more research on NoV might be needed.

In the present study, six specimens contained double infections, making up 2.2% (6/270) of the PCR-positive samples, and 12 (4.3%; 12/276) viral isolates in total were detected. Of these, GAR was most frequently detected (1.8%; 5/276). There were no triple infections, and Chung et al. (10) reported a multiple infection rate of 1.6%, lower than that in our study.

In the analysis of temporal patterns of isolation between July 2010 and October 2012, PCR positivity was highest in December (62.5%), followed by February (58.3%) and January (54.1%). Chung et al. (10) reported that positivity was highest in March, followed by February and January. Although their results were slightly different from ours, the trends are similar in that positivity was higher in the winter and early spring. Furthermore, our study showed that NoV-GII detection was highest in December, January, and February, and that GAR detection was highest in April, February, and March, indicating that these two viruses were detected mostly in the winter and early spring. Chung et al. (10) and Cheryl et al. (14) also reported that GAR was prevalent during January, February, and March. Additionally, Konno et al. (15) reported that positivity between December and February was 66%, and that between March and May was 56%, indicating few differences with our study.

In contrast to GAR being more prevalent in the past, NoV-GII detection has increased in the last 2 years, and these findings are similar to the reports by the KCDC. Furthermore, Shim (16) reported a recent increase in NoV infections. It was reported that NoV is the cause of 70–80% of epidemic enteritis in developed countries (17), and as NoV prevalence is increasing in Korea, its

importance is increasing to become on par with that of GAR (16). We believe that the incidence of NoV infections will continue to increase, and continued research will be necessary.

In this study, we demonstrated the performance of the simultaneous detection of five enteric viruses in clinical stool specimens using mPCR. We also reported the epidemiological study of enteric viral infections. These results could be helpful for the diagnosis and subsequent epidemiological surveillance of enteric virus infections.

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